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Relevance of trichothecenes in fungal physiology: Disruption of *tri5* in *Trichoderma arundinaceum*

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ABSTRACT

Trichothecenes are sesquiterpenoid mycotoxins produced mainly by *Fusarium* species. Harzianum A (HA), a non-phytotoxic trichothecene produced by *Trichoderma arundinaceum*, has recently been found to have antagonistic activity against fungal plant pathogens and to induce plant genes involved in defense responses. In the present work, we have shown that disruption of the *T. arundinaceum tri5* gene, which encodes a terpene synthase, stops the production of HA, alters the expression of other *tri* genes involved in HA biosynthesis, and alters the expression of *hmgR*, *dpp1*, *erg9*, *erg1*, and *erg7*, all genes involved in terpene biosynthetic pathways. An increase in the level of ergosterol biosynthesis was also observed in the *tri5* disrupted transformant in comparison with the wild type strain. The loss of HA also resulted in a drastic reduction of the biocontrol activity of the transformants against the phytopathogenic fungi *Botrytis cinerea* and *Rhizoctonia solani*. Finally, the effect of *tri5* gene disruption on the regulation and balance of intermediates in terpene biosynthetic pathways, as well as the hypothetical physiological role of trichothecenes, both inter- and intracellularly, on regulation and biocontrol, are discussed.

1. Introduction

Trichoderma is well known for the ability of some of its strains to act as important biocontrol agents against phytopathogenic fungi (Harman et al., 2004; Lorito et al., 2010). In interactions with plants, it has been shown that some *Trichoderma* strains can act as biofertilizers, as inducers of plant defense responses, and also can increase tolerance to abiotic stresses (Shoresh et al., 2010; Hermosa et al., 2012). Many *Trichoderma* strains produce primary or secondary metabolites and enzymes with diverse industrial interest. Among the metabolites produced by *Trichoderma,* trichothecenes have attracted attention in recent years because they are described as important mycotoxins, with phytotoxicity as well as animal and human toxicity (Desjardins, 2006; McCormick et al., 2011). *Trichoderma* species of the Brevicompactum clade, *Trichoderma brevicompactum* and *Trichoderma arundinaceum*, produce two different trichothecenes, trichodermin and Harzianum A (HA), respectively. While trichodermin is known to be phytotoxic, Harzianum A is not. Thus, HA serves as a model of a non-phytotoxic trichothecene produced by a fungal strain that shows a significant biocontrol activity (Malmierca et al., 2012), and can be used to study the impact of trichothecenes in fungal physiology.

The genes involved in the biosynthesis of trichothecenes (*tri* genes) in *T. brevicompactum and T. arundinaceum* have been recently cloned and characterized (Cardoza et al., 2011; Tijerino et al., 2011; Malmierca et al., 2012). It was shown that the *tri* genes are clustered, similar to that which occurs in *Fusarium*, but these two genera have important differences in the genetic organization of these genes. The *Fusarium tri* genes are located at three different *loci*, with the main *locus* including 12 genes with *tri5* located at a central position. The *tri5* gene in both *Fusarium* and *Trichoderma* encodes trichodiene synthase, a terpene cyclase that catalyzes the first step of the trichothecene biosynthetic pathway, converting farnesyl diphosphate (FPP) to trichodiene (Hohn and Van Middlesworth, 1986; Hohn and Desjardins, 1992; Cardoza et al., 2011). In contrast, the "main cluster" of *tri* genes in *Trichoderma* does not contain the *tri5* gene, and includes only seven genes with a marked difference in their relative organization in comparison with that of the *tri* genes of *Fusarium* (Cardoza et al., 2011). This is the only trichothecene producer described so far in which *tri5* is not located in the main *tri* cluster. In *Trichoderma*, trichodiene is oxygenated at the C2, C11, and C12 positions by the Tri4 protein (trichodiene monooxygenase) giving rise to isotrichodiol, which is non-enzymatically converted to 12,13-epoxytrichothecene (EPT),

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and afterwards oxygenated at C4 by the Tri11 protein (trichothecene monooxygenase) to produce trichodermol. The latter is finally acylated by the Tri3 protein (trichodermol acyltransferase) that adds a 2,4,6 octatriendioyl side chain to the C-4 hydroxyl group to produce HA (Fig. 3B) in *T. arundinaceum* or an acetyl to the C-4 hydroxyl group in *T. brevicompactum* to produce trichodermin (Cardoza et al., 2011; Tijerino et al., 2011). Thus, *tri5* and the gene/s hypothetically involved in the biosynthesis of the octatrienedioyl side chain, the latter of which has not yet been cloned, would be in other genomic locations.

Previously we described the characterization of a *tri4* gene disrupted transformant (Malmierca et al., 2012). Surprisingly, this transformant showed traces of HA production as well as production of 12,13-epoxytrichoene-2-ol derivatives, the production of which may have been due to one or more non-specific P450 oxygenases. In that *tri4* disrupted transformant, an increase in total chitinase activity and a reduction in biocontrol activity were detected. However, it was not determined if these observed results were due to the disruption of the *tri4* gene alone or from the production of the additional 12,13-epoxytrichoene-2-ol derivatives.

In the present work, we describe the isolation of a *T. arundinaceum* mutant carrying a disrupted *tri5* gene. This mutant allowed us to characterize the effect of a total lack of trichothecene production on several phenotypic characteristics of *Trichoderma*, as well as genotypic influences on the level of expression of other genes. Five genes in the terpene biosynthetic pathway were chosen for the study. *dpp1* is involved in the dephosphorylation of several isoprenoid phosphates, including FPP conversion to farnesol (Faulkner et al., 1999), a compound that acts as an important regulator of the cellular levels of FPP. *hmgR*, encodes hydroxymethylglutaryl CoA (HMG-CoA) reductase that catalyzes the production of mevalonate from HMG-CoA. This gene/enzyme is regulated at several levels, including transcription, translation, and enzyme degradation (Goldstein and Brown, 1990; Meigs and Simoni, 1997; Leichner et al., 2011). *erg9* encodes squalene synthase, the enzyme that is responsible for the first specific step of ergosterol biosynthesis, converting FPP into squalene. *erg9* has been described as a key target in the regulation of ergosterol biosynthesis (Asadollahi et al., 2010). *erg1* encodes squalene epoxidase, an enzyme involved in the oxygenation of squalene giving rise to oxidosqualene in a reaction that implies the entry of the squalene in an energy costly branch that produces ergosterol (Leichner et al., 2011). Lastly, *erg7* encodes oxidosqualene cyclase, which is involved in the cyclization of oxidosqualene to produce lanosterol (Corey et al., 1994).

Finally, we describe the effect of *T. arundinaceum tri5* disruption on antifungal activity.

2. Materials and methods

2.1. Strains, culture media, and culture conditions used in the present work

T. arundinaceum IBT 40837 (=Ta37) (IBT Culture Collection of Fungi at the Department of Biotechnology, Technical University of Denmark) was kindly provided by Ulf Thrane. For trichothecene analysis and RNA isolation, *T. arundinaceum* strains were grown using a two-step procedure in CM (0.5% malt extract, 0.5% yeast extract, 0.5% glucose) followed by growth in PDB modified medium as described previously (Cardoza et al., 2011). For ergosterol and squalene quantification, Ta37 and TaΔTri5 spores were inoculated in CM medium and incubated at 28 °C, 250 rpm, for 24 h. Then, 4 g (wet weight) of filtered mycelia were added to 100 ml of PDB modified medium and incubated at 28 °C, 250 rpm, for 24 and 96 h.

Phytopathogens *Botrytis cinerea* 98, isolated from diseased strawberry plants, *Rhizoctonia solani* CECT 2815, *Myrothecium roridum* ATCC 52485, and *Fusarium sporotrichioides* CECT 20166 were used as targets in the dual confrontation assays. *B. cinerea* and *R. solani* were also included in growth assays on membranes.

All fungal strains were routinely maintained on PDA (2.4% PDB, 2% agar), except *B. cinerea* and Ta37, which were maintained on MEA (2% glucose, 2% malt extract, 1% peptone, 2% agar, pH 5.6) and PPG (2% mashed potatoes, 2% glucose, 2% agar), respectively.

Three *Solanum lycopersicum* varieties: var. Marmande (Semillas Battle S.A., Barcelona, Spain), var. Tres Cantos and var. Muchamiel (Rocalba S.A., Girona, Spain) were used for fungal-plant interaction studies.

2.2. Construction of pΔtri5 and pUStri5 plasmids

A 784 bp fragment (GenBank accession number FR715494) of the *tri5* gene (from 54 to 837 bp) of Ta37 was amplified using iProof High-Fidelity DNA Polymerase (BioRad, Hercules, CA) and primer pair 2100/2101 (Table S1a, Supplementary data) (the latter primer with an *Asc*I restriction site at the end) using genomic DNA as template. The amplicon was band-purified with the Ultraclean DNA purification kit (MoBio, Carlsbad, CA) and cloned into pCR™4 Blunt-TOPO® (Invitrogen, Carlsbad, CA). The resulting plasmid was cut with *Asc*I and ligated with a chimeric hygromycin B gene (2.5 kb) (Turgeon et al., 1987), which contained *Asc*I restriction sites at both ends of the chimera, leading to the final 7.3 kb pΔtri5 vector (Fig. S1A, Supplementary data).

For construction of the pUStri5 plasmid, intact *tri5* was amplified by PCR using *Pfu* polymerase and the primer pair 2114/2115 (Table S1a, Supplementary data). The 1.2 Kb DNA fragment was gel purified and cloned into the *Bam*HI restriction site of pAN52.1 (Punt et al., 1987). The resulting plasmid was digested with *Stu*I and *Hind*III and the 3.8 Kb band that contained the *tri5* expression cassette was gel purified and cloned into the *Eco*RI restriction site of pUSR0 (Cardoza et al., 2006a), thus obtaining the 17.1 Kb plasmid pUStri5 with the phleomycin resistance gene as selectable marker (Fig. S2B, Supplementary data).

2.3. Transformation of T. arundinaceum

The host fungus was transformed with plasmid pΔtri5 using a protoplast transformation protocol as described previously (Malmierca et al., 2012). Transformants were selected by hygromycin B (100 μg/ml) resistance. The selected transformants were analyzed by PCR using the primer pairs 2114/2115 and 2114/T7 (Table S1a, Supplementary data) using the Terra™ PCR Direct Polymerase Mix (Clontech, Mountain View, CA) and by Southern hybridization to detect those with the pΔtri5 vector inserted into the *tri5* gene.

Strain TaΔTri5 was transformed with pUStri5 plasmid by using *Agrobacterium*-mediated transformation as described previously (Cardoza et al., 2006a). The transformants were selected by phleomycin (30 μg/ml) resistance, and after two rounds of growth in selective medium, transformants were analyzed by PCR using the primer pair 2114/pAN52-trpC (Table S1a, Supplementary data) and the same procedures as above.

2.4. Extraction and chemical analysis of HA

Cultures of wild-type and transformants were analyzed by high-performance liquid chromatography (HPLC) for HA as previously described (Cardoza et al., 2011).

2.5. Antifungal assays

2.5.1. Direct confrontation assay

In vitro confrontation assays between *Trichoderma* strains (Ta37 and the disruptant strain TaΔTri5) and the pathogens *R. solani*,

M. roridum, and *F. sporotrichioides* (on PDA plates), and *B. cinerea* (on MEA plates), were established as described in Malmierca et al. (2012). Assays were performed in triplicate, and single cultures of *Trichoderma* strains and pathogens were used as control. Photographs were taken after 10 days.

2.5.2. Growth assay on membranes

To test the effect of *Trichoderma* metabolites on the growth of selected fungal plant pathogens, 5-mm-diameter PDA plugs of Ta37 or disrupted strain TaΔTri5 were placed at the center of Petri dishes containing PDA or MEA medium, on cellophane sheets (Shengzhou Pengyu Trading Co, Ltd., Zhejiang, China) or 10 kDa-cut-off dialysis membranes (Sigma, St. Louis, MO). After 2 days of incubation at 28 °C, the membranes were removed from the plates and a single 5-mm diameter mycelial plug of the pathogen *R. solani* on PDA plates and *B. cinerea* on MEA plates was placed at the center of the dish. In parallel, the pathogens were grown on PDA or MEA (control plates). Each pathogen was tested on three plates. Growth diameters were measured after 72 and 96 h for *R. solani* and *B. cinerea*, respectively. The results are expressed as percentages of growth inhibition of each pathogen by *Trichoderma* strains.

To test the permeability of each type of membrane to HA, control and test plates were prepared. Control plates were made by adding 40 μl of acetonitrile over MEA plates with either cellophane or dialysis membranes. When the surface of the plate was dry, either no *Trichoderma* plug was added, or an agar plug of Ta37 or TaΔTri5 was added. After 2 days of incubation at 28 °C, the membranes were removed and a single 5-mm diameter mycelia plug of *B. cinerea* was placed in the center of the dish. Growth diameter was measured after 72 h. For the test HA plates, 30 μg of HA (dissolved in acetonitrile, 40 μl total volume) was added onto the membrane, allowed to dry, and then a plug of Ta37 or TaΔTri5 was added. After 2 days of incubation, the membranes were removed and a plug of *B. cinerea* was placed on the center of the plate. Growth was determined as above. Comparisons were calculated between the test plates and the control plates. All plates were done in triplicate.

2.6. Tomato plant assays

Surface sterilized tomato seeds were planted in commercial loamy field soil [Kekkilä 50/50 (Projar S.A., Valencia, Spain), refer to Malmierca et al. (2012) for composition]. Pots were incubated in a greenhouse at 22 ± 4 °C, and watered as needed. Eighteen day-old leaves were cut and placed in Petri dishes with sterile wet filter paper on the bottom. Then, 15 μl of *B. cinerea* conidial suspensions (5×10^5 spores/ml) in germination buffer (20 mM glucose and 20 mM KH_2PO_4) were placed onto the leaf surface. After approximately 4 h (when the *B. cinerea* conidial suspension was dry) 15 μl of Ta37 or TaΔTri5 filter sterilized broth (or PDB medium in control assays) were placed over the conidial suspension spot and the dishes were incubated in a greenhouse at 22 °C for 96 h. Three inoculations were made per leaf on twelve leaves per treatment and three replicates for each experiment. The necrotic lesions were measured every 24 h.

2.7. Chitinase activity assays

Cultures of the different strains were grown in modified PDB medium at 28 °C, 250 rpm, for 96 h. Supernatant samples of 8 ml were taken every 24 h. The extracellular chitinase activity was quantified using *p*-NP-β-D-glucosaminide (*p*-NPβDG) (Sigma, St. Louis, MO) as substrate as previously described in Malmierca et al. (2012).

2.8. Assay of hydroxymethylglutaryl Coenzyme A reductase activity

HMG-CoA reductase was measured as described by Servouse and Karst (1986) and Cardoza et al. (2007) with slight modifications. The mycelia of all strains were ground with liquid nitrogen in a mortar, the powder was resuspended in 0.1 M sodium phosphate buffer, pH 7.0, containing 0.5% Triton-X100, 1 mM EDTA, 10 mM dithiothreitol (DTT), and a protease inhibitor cocktail containing: 0.5 mM 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF), 2.5 mM 1,10-phenanthroline, 11 μM pepstatin A, and 7 μM of cysteine protease E-64 (Sigma, St. Louis, MO). The homogenate was incubated for 90 min at 0 °C, centrifuged at 1000 g for 5 min, and the resulting supernatant was centrifuged again at 8000 g for 15 min.

The reaction mixture contained, in 0.5 ml of assay buffer (assay buffer was the homogenization buffer without protease inhibitors), 150 μM NADPH and 100 μl of the supernatant. Endogenous oxidation of NADPH was recorded at 340 nm, with a Varian Cary 50 spectrophotometer before starting the reaction with 100 μM of HMG-CoA. The reaction was incubated at 30 °C and the specific activity was calculated as nmol of NADPH oxidized per min per mg of protein.

Protein concentration was determined by the Bradford method using ovalbumin as standard (Bradford, 1976).

2.9. Fragment cloning of *T. arundinaceum dpp1, hmgR, erg1, erg7, and erg9 genes*

One orthologue to *dpp1*, encoding diacylglycerol pyrophosphate phosphatase, was found on each of the available *Trichoderma* genomes of *Trichoderma reesei* QM6a, *Trichoderma atroviride* IMI206040, and *Trichoderma virens* Gv29-8. Oligonucleotides designed from conserved regions of the *dpp1* genes from these fungi (Table S1b, Supplementary data), were used to clone an internal fragment of the *T. arundinaceum* orthologue gene. The resulting sequence, of 364 bp, contains two introns of 71 and 88 bp and encodes for a region of 68 amino acids of the DPP1 protein. This region showed a 91.0, 88.2, 86.8, and 35.7% similarity with the *T. reesei*, *T. atroviride*, *T. virens*, and *S. cerevisieae* DPP1 proteins, respectively.

Fragments of *T. arundinaceum hmgR*, *erg1*, and *erg7* genes, encoding the HMG-CoA reductase (HMGR), squalene epoxidase (ERG1), and oxidosqualene cyclase (ERG7) respectively, were cloned by PCR using degenerate oligonucleotides based on the available sequences of these genes from *Trichoderma harzianum* (Cardoza et al., 2006b, 2007) (Table S1b). As a result, a 675 bp fragment of the Ta37 *hmgR* gene was cloned. This fragment, without introns, encodes a region of 225 amino acids of the HMGR protein, showing an 81.5, 80.7, 77.0, and 75.3% similarity with the *T. harzianum*, *T. virens*, *T. atroviride*, and *T. reesei* HMGR proteins respectively. Secondly, a 408 bp fragment without introns, corresponding to the Ta37 *erg1* gene was cloned and encodes for a region of 136 amino acids of the ERG1 protein which showed similarities of 95.6, 94.9, 92.6, and 89.0%, when compared with its orthologues in *T. harzianum*, *T. reesei*, *T. virens*, and *T. atroviride* respectively. In parallel, a 618 bp fragment, internal to the Ta37 *erg7* gene, was cloned and sequenced. The 206 amino acids of the ERG7 protein encoded by this region showed a similarity of 90.8% with the protein of *T. atroviride* and of 87.9% with its orthologues in *T. harzianum*, *T. reesei*, and *T. virens*.

Finally, one orthologue to *erg9*, encoding the squalene synthase, was found on the three *Trichoderma* genomes available. Oligonucleotides based on the conserved regions of the *erg9* genes from these fungi (Table S1b), were designed and used to clone an internal fragment of the *T. arundinaceum* orthologue gene. The resulting fragment, of 590 bp without introns, encodes a region of 196

amino acids that showed an 89.8, 87.8, and 86.2% similarity with the ERG9 proteins from *T. reesei*, *T. virens* and, *T. atroviride* respectively.

These internal fragment sequences, once they were confirmed to correspond to the predicted genes, were used to design oligonucleotides (Table S1c, Supplementary data) to study the relative level of expression of their corresponding genes in the TaΔTri5 mutant in comparison with the wild type Ta37 strain, by qPCR.

2.10. Ergosterol and squalene quantification

Total intracellular sterols were extracted and ergosterol and squalene content were calculated as reported previously by Cardoza et al. (2007) and Ghimire et al. (2009), respectively. All measurements were made in triplicate.

2.11. Inhibition of Botrytis spore germination assay

To determine if culture broths of the different *Trichoderma* strains affect the germination of spores of *B. cinerea*, we tested several volumes (ranging from 5 to 25 μl) of filter sterilized supernatants from Ta37 or TaΔTri5 culture in a microtiter plate. Each well was filled with the desired volume of the *Trichoderma* broths, 5 μl of *B. cinerea* spores (containing 300 spores) in germination buffer, and PDB medium up to a final volume of 100 μl. The microtiter plate was covered by a film to maintain high humidity and incubated in darkness at 22 °C overnight. Spores/germlings were observed under 4 × magnification. Each combination was performed in triplicate and three independent experiments were made.

2.12. Nucleic acids extraction and manipulation

The procedures for fungal genomic DNA isolation and for Southern hybridization were performed as described previously (Cardoza et al., 2011).

To isolate total RNA, *Trichoderma* strains were grown as indicated above. Then, mycelia were recovered from PDB modified medium by filtration, washed with 0.9% NaCl, dried on absorbent filter paper, frozen with liquid nitrogen, and ground in a mortar. RNA was extracted with the phenol-SDS method (Ausubel et al., 1987) and treated with RNase protector and DNase (Fermentas, Vilnius, Lithuania).

The cDNA was synthesized using one μg of total RNA and a Reverse Transcription System using an Oligo$(dT)_{15}$ primer (Promega, Madison, WI).

Labelling, hybridization, and immunological detection were carried out with a nonradioactive labelling and immunological detection kit with CDP-Star as the chemiluminescent substrate (Roche, Mannheim, Germany), as previously described (Cardoza et al., 2007).

2.13. qPCR experiments

In order to perform comparative studies of the *T. arundinaceum* gene expression levels, oligonucleotides were designed based on the sequences of the studied genes (Table S1c, Supplementary data). In *T. arundinaceum*, according to the GeNorm software (Vandesompele et al., 2002) results, α-actin and *gpd* were used as reference genes. For the sequence of oligonucleotides used in the qPCR analysis of *T. arundinaceum tri* and housekeeping genes, see Malmierca et al. (2012). The qPCR reactions were carried out using Step One Plus™ (Applied Biosystems, USA). The reactions were performed in a total volume of 20 μl: 10 μl Power SYBR® Green PCR Master Mix (Applied Biosystems, USA), 0.4 μl Forward Primer 10 μM, 0.4 μl Reverse Primer 10 μM, 5 μl cDNA, and H_2O to 20 μl. The REST 2009© software (Pfaffl et al., 2002) was used to calculate the relative expression values and the significance of the differences between the gene expression levels. For each primer pair used in this work, we performed a standard curve with 320, 160, 80, 40, 20, and 10 ng cDNA to determine the PCR amplification efficiency (E value). Each measurement was made in triplicate.

2.14. Statistical analysis

ANOVA and Mann–Whitney U tests were performed with IBM SSPS Statistics 19 Software.

2.15. Nucleotide sequence accession numbers

Sequences of Ta37 obtained in the present work have been deposited in the GenBank database. Accession numbers are as follows: HE866933 for *dpp1*, HE965020 for *hmgR*, HE965019 for *erg1*, HE974352 for *erg7*, and HF548663 for *erg9*.

3. Results

3.1. Disruption of tri5 affects phenotype and blocks production of HA

Plasmid pΔtri5 (Fig. S1A, Supplementary data) was transformed into Ta37 to obtain a *tri5*-disrupted strain. Up to 200 hygromycin B-resistant transformants were analyzed by PCR using oligonucleotide pair 2114/2115 (Table S1a), which amplified a 1.2-kb fragment in a nondisrupted transformant and an 8.5-kb fragment in a TaΔTri5 disruptant, and with primer pair 2114/T7 (Table S1a) that amplifies a 0.9 kb band only in the *tri5*-disrupted mutant. Only transformant #182 displayed the expected result (Fig. 1).

This TaΔTri5 transformant displayed significant differences in its phenotypic characteristics, in comparison with its wild type counterpart, as it showed a faster mycelial growth rate when it was grown on solid MMT or PDA media. However, similar growth rates were observed when both strains were grown on PPG medium (Table 1). Additionally, on PPG medium, the *tri5*-disrupted transformant showed a significant delay in the onset of sporulation in comparison with the wild type strain (Fig. 2).

No HA was detected in the TaΔTri5 strain culture medium (Fig. 3A), whereas the wild-type strain showed a maximum specific production of 18.6 μg HA/ml supernatant/mg dry weight at 24 h of culture (Fig. 3C).

3.2. Complementation of tri5 gene mutation restores the HA production phenotype

Strain TaΔTri5 was transformed with plasmid pUStri5 which contains a cassette of phleomycin resistance as a marker (the TaΔTri5 mutant is resistant to hygromycin). As a result, 52 phleomycin/hygromycin resistant transformants were isolated. Transformants #1 to #25 were analyzed by PCR and 24 of them gave the expected amplification band (2.0 kb). Ten transformants (#15 to #24) were studied to determine their ability to produce HA in 24 h PDB grown cultures. Of these, three transformants produced detectable levels of HA. Transformants #17 and #18 produced low levels of HA (0.128 and 0.084 μg HA/ml supernatant/mg dry weight respectively) while transformant #15 produced 13.96 μg HA/ml supernatant/mg dry weight. Genomic DNAs from these strains, including transformant #16 as an HA non-producing control, were isolated and digested with the restriction endonuclease *Pvu*II. The digested DNAs were then hybridized with a 1.2 kb probe (see Fig. S2A). All the transformants gave the expected bands of 1.3 and 1.5 kb, belonging to the complementation cassette, and an additional band of 1.1 that corresponded to the 3′ region of the endogenous *tri5* gene (See Fig. S2A and C). The 1.3 kb band of the

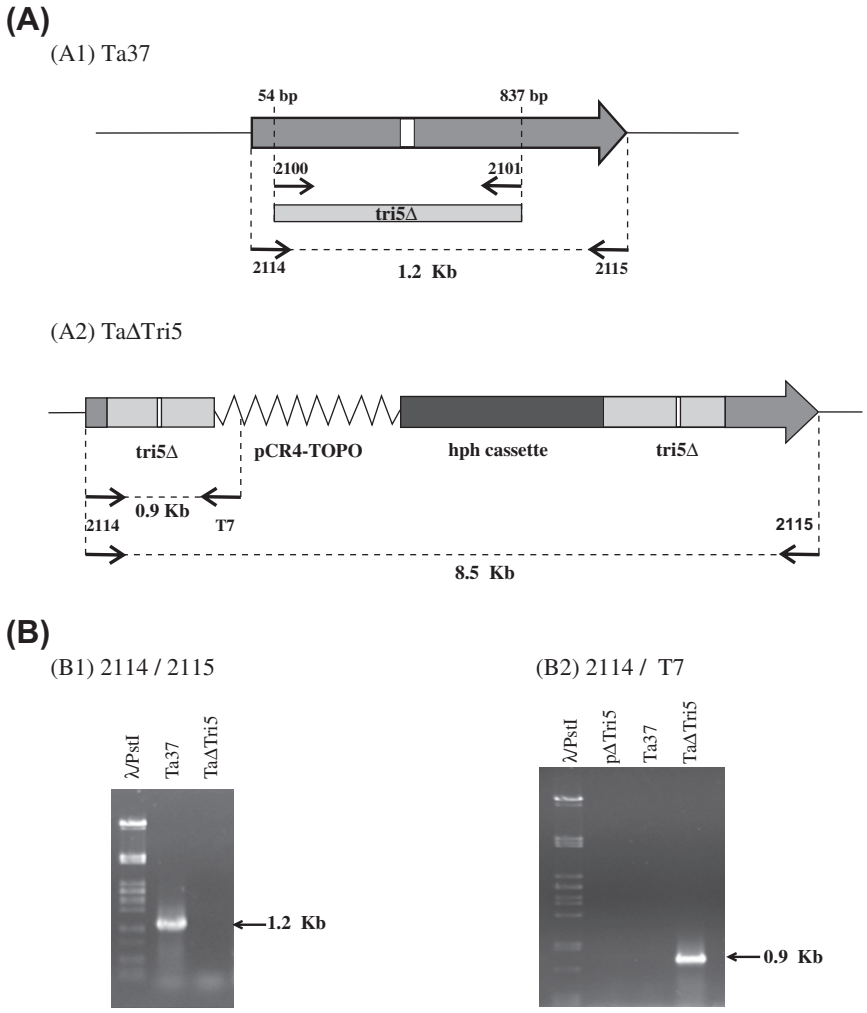


Fig. 1. (A) *tri5* region in the genome of Ta37 (A.1) and TaΔTri5 (A.2). White box represents the *tri5* gene intron. Oligonucleotides used to analyze the transformants are indicated by small arrows. The relevant fragments are delineated with dotted lines including their sizes. (B) PCR analysis of the TaΔTri5 mutant in comparison with the Ta37 strain. The plasmid pΔtri5, used in the disruption experiment, was introduced as a control in the PCR experiment shown in panel B.2. *Note*: In the PCR experiment shown in panel B.1, the TaΔTri5 genomic DNA gave a negative result since the polymerization time used in this experiment was specific for amplification of the 1.2 kb fragment of the wild type strain and not that of the 8.5 kb fragment expected in the *tri5*-disrupted mutant.

Table 1
Growth of Ta37 and TaΔTri5 strains in three different culture media.

	PPG		PDA		MMT	
	Ta37	TaΔTri5	Ta37	TaΔTri5	Ta37	TaΔTri5
48 h	$3.50^{a} \pm 0.20$	$3.50^{a} \pm 0.10$	$2.70^{a} \pm 0.00$	$3.00^{b} \pm 0.10$	$0.57^{a} \pm 0.03$	$2.37^{b} \pm 0.06$
72 h	$7.13^{a} \pm 0.30$	$7.30^{a} \pm 0.10$	$4.73^{a} \pm 0.06$	$5.53^{b} \pm 0.06$	$1.13^{a} \pm 0.15$	$5.43^{b} \pm 0.06$
96 h	$8.50^{a} \pm 0.00$	$8.50^{a} \pm 0.00$	$6.90^{a} \pm 0.10$	$8.27^{b} \pm 0.06$	$2.20^{a} \pm 0.10$	$8.40^{b} \pm 0.10$
120 h	$8.50^{a} \pm 0.00$	$8.50^{a} \pm 0.00$	$8.27^{a} \pm 0.06$	$8.50^{b} \pm 0.00$	$3.37^{a} \pm 0.15$	$8.50^{b} \pm 0.00$

The numbers indicate the diameter, in cm, of the colonies.
$n = 3$, Mann–Whitney U test.
[a,b] For each culture medium and time point values followed by different superscript letters are significantly different ($p < 0.001$).

tri5 endogenous copy could not be distinguished in the transformants because it was the same size as the 1.3 kb band of the complementation cassette.

3.3. Interruption of tri5 gene affects the expression of other tri genes

All *tri* genes, except *tri4*, are expressed at a higher level in the TaΔTri5 transformant in comparison with the wild type strain (Fig. 4). *tri11* and *tri3*, which encode the trichothecene C4-monooxygenase and trichodermol C4-acyltransferase respectively, the two structural biosynthetic enzymes following Tri4 in the HA biosynthetic pathway, showed a higher ratio of expression [3.37 ($p = 0.000$) and 4.825 ($p = 0.026$) fold, respectively] (Fig. 4). In this study, it was possible to determine the effect of the *tri5* gene disruption on its own expression, since the construction used to disrupt this gene did not affect the promoter region or the 5′ end of

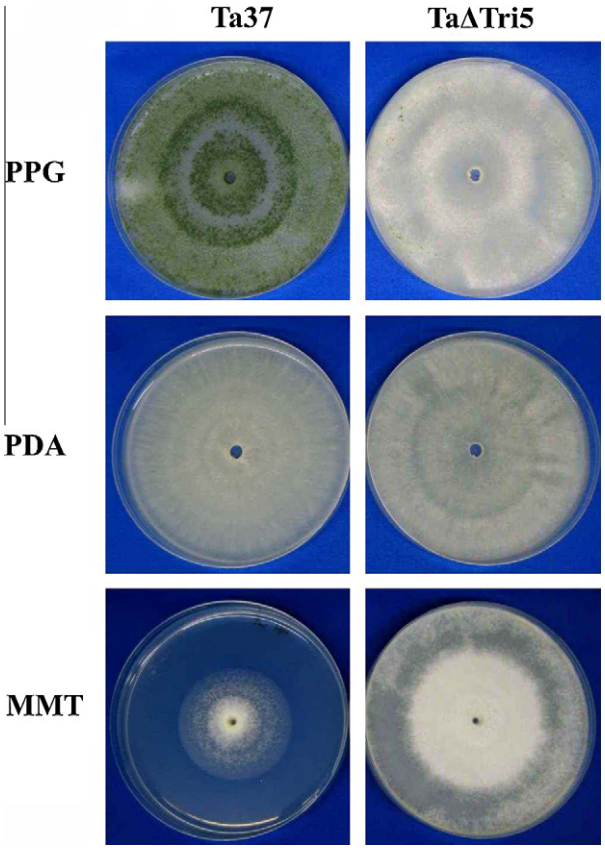


Fig. 2. Growth of Ta37 (wild type) and TaΔTri5 strains on PPG, PDA, and MMT after 6 days of incubation at 28 °C. On PPG, spores of Ta37 strain are visualized in black bands.

the *tri5* gene (where the qPCR primers were located). As a result, the expression of the *tri5* gene was slightly induced by a factor of 1.581 ($p = 0.000$) in the TaΔTri5 mutant when compared with the Ta37 strain.

3.4. Expression of hmgR, dpp1, erg9, erg1, and erg7 genes

The effect of the *tri5* gene disruption on the expression of other key genes belonging to the terpene pathway was analyzed. qPCR determined the relative expression of *hmgR*, *dpp1*, *erg9*, *erg1*, and *erg7* between the TaΔTri5 mutant and the wild type Ta37 strain. When 24 h grown mycelia were analyzed, an up-regulation of *hmgR*, *dpp1*, and *erg9* genes by factors of 3.927 ($p = 0.031$), 3.877 ($p = 0.039$), and 2.503 fold ($p = 0.031$), respectively, was observed.

However, when the comparative level of expression of these genes was determined from mycelia grown for 96 h, it was detected that the expression of *hmgR*, *erg9*, *erg1*, and *erg7* genes was down-regulated by factors of 0.296 ($p = 0$), 0.505 ($p = 0.038$), 0.301 ($p = 0$), and 0.156 ($p = 0.034$) respectively, while the expression of *dpp1* was not significantly affected (Fig. 5).

3.5. tri5 Gene interruption increases the hydroxymethylglutaryl-CoA reductase (HMGR) activity

In order to determine if the *tri5* gene disruption affected the general balance of the terpene biosynthetic pathway (see Fig. 8), HMGR activity was quantified. This enzyme catalyzes the synthesis of mevalonate from HMG-CoA and is strongly regulated at various levels. We would expect HMGR activity to vary if the lack of processing of FPP (the precursor of ergosterol and HA) toward HA in the TaΔTri5 mutant caused the accumulation of sterols and/or other terpenoid compounds. The TaΔTri5 transformant showed an increase in the level of HMGR activity of 92.4% and 23.9% in comparison with the transformed control strain (without insert) at 24 and 96 h of culture respectively (Table 2).

3.6. Production of ergosterol and squalene

The TaΔTri5 transformant showed significantly higher levels of ergosterol production when compared with the Ta37 strain in liquid PDB cultures grown for 24 and 96 h, with the highest difference observed in 24 h samples (+85.44%) ($p < 0.05$). The production of squalene by the TaΔTri5 mutant was also higher than the wild type strain in samples from 24 h cultures (+88.5%) ($p < 0.05$) but the levels of this triterpene intermediate were not statistically different between the *tri5* mutant and the wild type strain in samples from 96 h cultures (Table 3).

3.7. Effect of the tri5 gene disruption on the chitinase activity

The level of chitinase activity, involved in the mycoparasitic activity of *Trichoderma*, in the *tri5*-disrupted strain was lower (statistically significant) at 48 and 72 h of growth when compared to such activity in the control strain (Table 4). These results are in contrast with the significantly higher levels of chitinase activity observed in the *tri4*-disrupted strain (Malmierca et al., 2012).

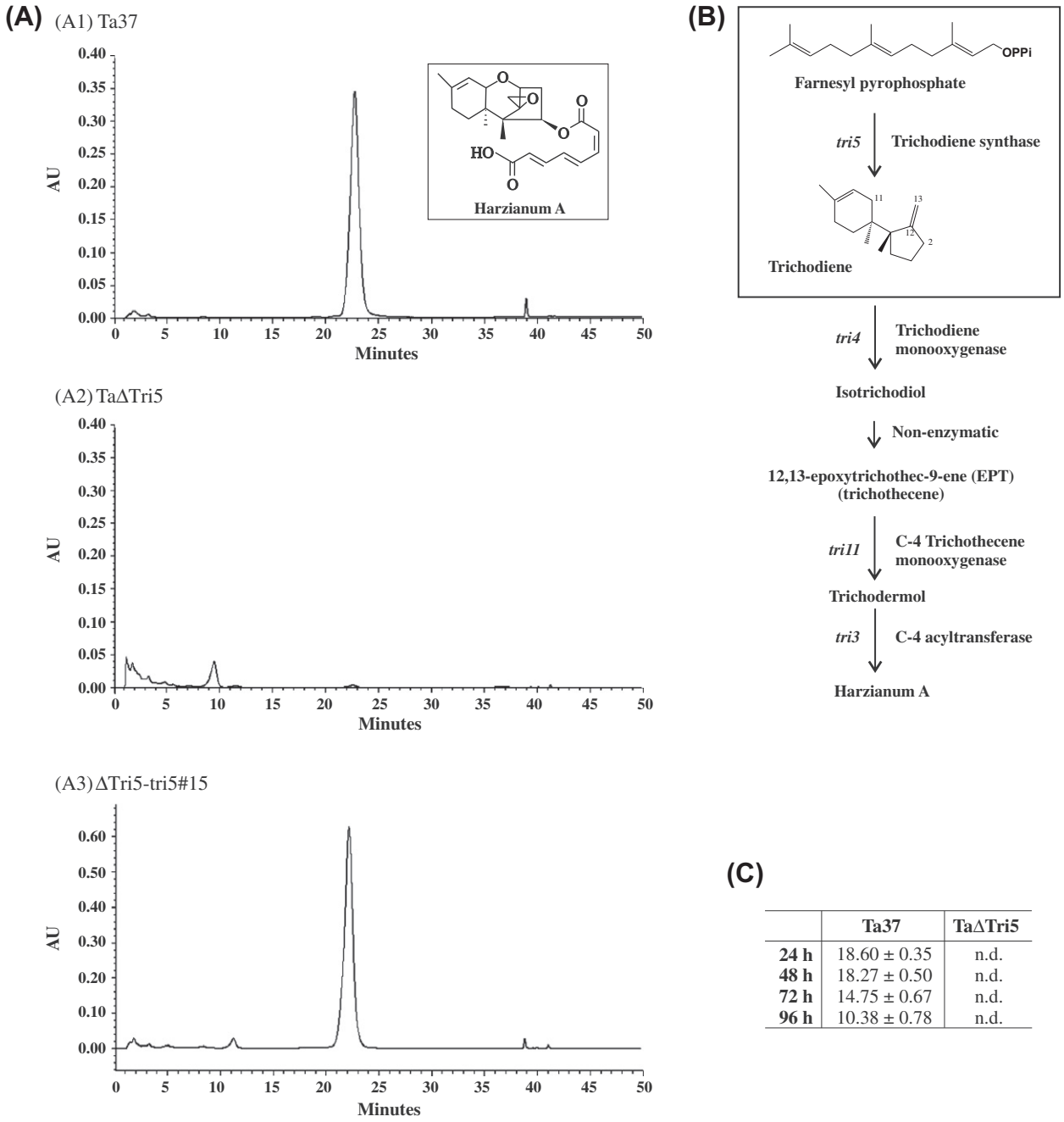
3.8. The tri5 disrupted transformant shows a reduction in antifungal activity against fungal pathogens

3.8.1. Growth on membranes

A membrane assay was used to measure the effect of *Trichoderma* metabolites, including HA, on the growth of selected fungal plant pathogens. Wild type *T. arundinaceum* had significant antifungal activity against *B. cinerea* and *R. solani* (Fig. 6) whereas the TaΔTri5 transformant had a significant reduction in this antifungal activity, suggesting that HA plays an important role in the mycoparasitic activity of *T. arundinaceum*.

To examine the effect of large *versus* small molecular weight metabolites, cellophane membranes (allowing small and large metabolites to pass through) were compared with dialysis membranes having a MW cutoff of 10 KDa (allowing only metabolites <10 KDa to pass through). The reduction of the antifungal activity in the TaΔTri5 strain against the two phytopathogenic fungi was greater when using dialysis membranes (cutoff, 10 kDa) than with cellophane (Table 5a). On the dialysis membranes, the reduction in the biocontrol activity, as measured by growth inhibition activity (RI = radial inhibition), in the TaΔTri5 transformant against *B. cinerea* was 42.4% [88.8% (% seen with Ta37/dialysis) – 46.4% (% seen with TaΔTri5/dialysis)] and 32.3% against *R. solani*. In the assays using cellophane membranes, the observed reduction of pathogen growth was lower than that obtained with dialysis membranes, suggesting that since HA has a MW of 0.4 kDa, a metabolite larger than 10 kDa also contributes to the *B. cinerea* and *R. solani* growth inhibition.

To check the permeability of the two different types of membranes to HA, 30 μg HA (dissolved in acetonitrile) in a 40 μl total volume were spread on each membrane, the membrane was lifted off after 2 days, and an agar plug of *B. cinerea* was inoculated. Results are shown in Table 5b, in which it can be observed that HA does permeate both membranes. Exogenous HA inhibits growth of *B. cinerea* by 51.74 or 59.61% in cellophane or dialysis assays respectively (when comparing control plates with and without exogenous HA). Approximately the same percentage (52.15%) was obtained in TaΔTri5 cellophane plates while in dialysis plates the value (73.64%) was higher. Finally, in assays using the Ta37 strain, no significant differences were detected when exogenous



(C)

	Ta37	TaΔTri5
24 h	18.60 ± 0.35	n.d.
48 h	18.27 ± 0.50	n.d.
72 h	14.75 ± 0.67	n.d.
96 h	10.38 ± 0.78	n.d.

Fig. 3. (A) HPLC chromatograms of extracts from Ta37 (wild type), TaΔTri5 (disruptant) and ΔTri5-tri5#15 (add-back) strains, (B) schematic representation of the HA biosynthetic pathway in which the reaction catalyzed by Tri5 protein has been boxed and (C) specific HA production in PDB medium (μg HA/ml supernatant/mg dry weight) by the *T. arundinaceum* strains used in the present work.

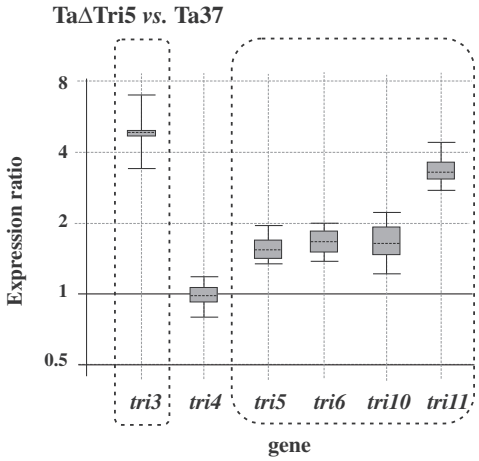
HA was added, because the HA produced by Ta37 is much higher than that exogenously added, thus no effect on the total RI can be seen (Fig. S3, Supplementary data).

3.8.2. Direct confrontation assays

Plate confrontation experiments between Ta37 or TaΔTri5 and the pathogens *B. cinerea*, *R. solani*, *M. roridum*, and *F. sporotrichioides* were carried out at 28 °C and plates were photographed after 10 days of incubation (Fig. S4, Supplementary data). In all cases, the four pathogens completely covered the surface after this incubation time on control plates of PDA or MEA. In the confrontation plates, *Trichoderma* strains overgrew the colonies of *B. cinerea*, *R. solani*, and *F. sporotrichioides*, and surrounded the colonies of *M. roridum*. Both *Trichoderma* strains (wild type Ta37 and TaΔTri5 mutant) were able to inhibit the growth of the four pathogens tested reducing the colony diameter of *B. cinerea* and *M. roridum* to no more than 25 mm. However, TaΔTri5 was significantly less efficient than Ta37 in the confrontation assays against *B. cinerea*.

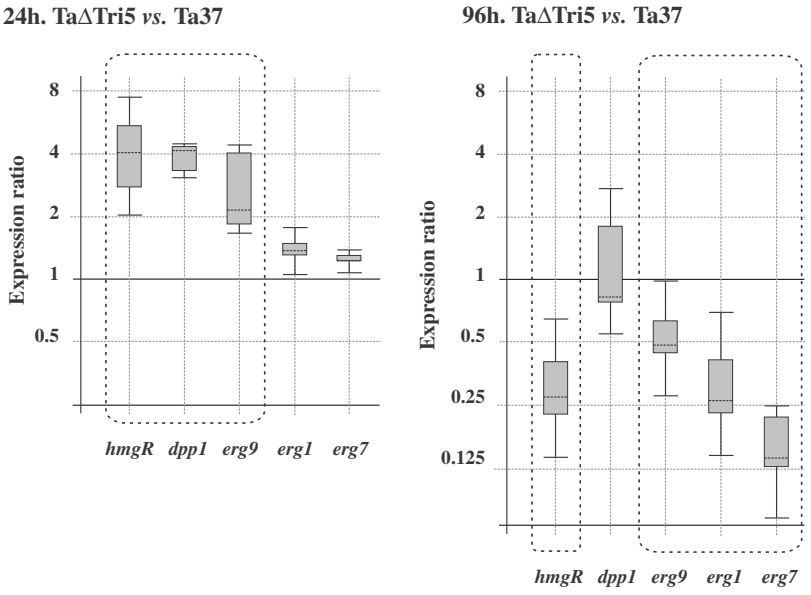
3.8.3. Effect of the culture broths from Ta37 and TaΔTri5 on B. cinerea spore germination

Ta37 culture broths inhibit *B. cinerea* spore germination more efficiently than the culture broths from the TaΔTri5 mutant (Fig. S5, Supplementary data), suggesting that HA is responsible for that inhibition since the only deduced difference between the *tri5*-disrupted transformant and the Ta37 strain is the production of this trichothecene.



	tri3	p(H$_1$)	*tri4*	p(H$_1$)	*tri5*	p(H$_1$)	*tri6*	p(H$_1$)	*tri10*	p(H$_1$)	*tri11*	p(H$_1$)
TaΔTri5 *vs.* Ta37	4.825*	0.026	0.988	0.916	1.581*	0.000	1.676*	0.000	1.683*	0.041	3.370*	0.000

Fig. 4. qPCR analysis of the comparative expression level of the *tri* genes in the TaΔTri5 transformant *versus* the level of expression of these genes in the Ta37 strain. The expression ratios as well as the statistical significance were calculated using the REST2009 program. The numeric data are illustrated at the bottom of the figure and those statistically significant ($p < 0.05$) are indicated with an asterisk and are boxed in the graphic representation.



	hmgR	p(H$_1$)	*dpp1*	p(H$_1$)	*erg9*	p(H$_1$)	*erg1*	p(H$_1$)	*erg7*	p(H$_1$)
24h. TaΔTri5 *vs.* Ta37	3.927*	0.031	3.877*	0.039	2.503*	0.031	1.376	0.058	1.237	0.050
96h. TaΔTri5 *vs.* Ta37	0.296*	0.000	1.074	0.854	0.505*	0.038	0.301*	0.000	0.156*	0.034

Fig. 5. Expression of *hmgR*, *dpp1*, *erg9*, *erg1*, and *erg7* genes in TaΔTri5 in comparison with Ta37 measured from mycelia grown 24 and 96 h. The comparisons and statistical analysis were performed using the REST2009 program. The numeric data are illustrated at the bottom of the figure and those showing differences which are statistically significant ($p < 0.05$) are indicated with an asterisk and are boxed in the graphic representation.

3.8.4. In vitro assay of tomato leaf lesions caused by B. cinerea in the presence of T. arundinaceum broth

When Ta37 broth was applied to leaves together with the pathogen inoculum, the production of *B. cinerea* lesions was totally abolished in the tomato varieties Tres Cantos and Muchamiel, while in the variety Marmande only partial inhibition was observed. The use of TaΔTri5 broth gave rise to lesions in all three tomato varieties tested, although they were significantly smaller ($p < 0.05$) than in the leaves treated only with *B. cinerea* conidia (Fig. 7 and Table 6), indicating that the broth of the TaΔTri5 mutant reduced the size of the lesion but failed to prevent lesion formation by *B. cinerea.*

4. Discussion

The disruption of the *tri5* gene in *T. arundinaceum* resulted in the total absence of the trichothecene HA without production of

Table 2
Specific HMG-CoA reductase activity (nmol/mg prot) of Ta37-p* and TaΔTri5 strains.

	24 h	% variation	96 h	% variation
Ta37-p*	26.9[a] ± 9.13	–	33.8[a] ± 2.29	–
TaΔTri5	51.0[b] ± 1.13	+92.4%	41.9[b] ± 2.52	+23.9%

*Ta37-p is the control strain obtained from Ta37 transformed with a plasmid without insert.
$n = 3$, Mann–Whitney U test.
[a,b] For each time point values followed by different superscript letters are significantly different ($p < 0.05$).

Table 3
Quantification of ergosterol and squalene production by Ta37 and TaΔTri5 strains in samples from 24 and 96 h cultures in PDB. % Variation of produced ergosterol and squalene between the wild-type and mutant are given.

		Squalene (mgS/g DW)	% Variation	Ergosterol (mgE/g DW)	% Variation
24 h	Ta37	0.200[b] ± 0.002		1.065[b] ± 0.045	
	TaΔTri5	0.377[a] ± 0.052	+88.5%	1.975[a] ± 0.085	+85.44%
96 h	Ta37	0.308[a] ± 0.046		1.705[b] ± 0.175	
	TaΔTri5	0.385[a] ± 0.034	–	2.870[a] ± 0.260	+68.32%

$n = 4$, ANOVA.
[a,b]For each time point, values followed by different superscript letters are significantly different ($p < 0.05$).

Table 4
Chitinase activity against *p*-NP-β-D-glucosaminide (nmol/min/mg DW) measured at four times in culture broths of Ta37-p* and TaΔTri5 strains.

	24 h	48 h	72 h	96 h
Ta37-p*	0.05[a] ± 0.0	0.84[a] ± 0.07	2.17[a] ± 0.06	4.65[a] ± 0.96
TaΔTri5	0.06[b] ± 0.0	0.18[b] ± 0.03	1.79[b] ± 0.14	4.24[a] ± 0.07

Ta37-p* is a control strain obtained from Ta37 transformed with a plasmid without insert.
[a,b] For each time point, values followed by different letters are significantly different (Mann–Whitney U test).

Table 5a
Percentages of radial growth inhibition (RI) of *Botrytis cinerea* and *Rhizoctonia solani* by *T. arundinaceum* Ta37 and TaΔTri5, grown on cellophane or dialysis (cut-off 10 kDa) membranes.

	B. cinerea (72 h)		*R. solani* (96 h)	
	Cellophane	Dialysis	Cellophane	Dialysis
Ta37	100.0[a] ± 0.0	88.8[a] ± 0.3	100.0[a] ± 0.0	100.0[a] ± 0.0
TaΔTri5	73.5[b] ± 1.0	46.4[b] ± 1.2	95.2[b] ± 2.1	67.7[b] ± 5.1

[a,b] For each column, values followed by different superscript letters are significantly different ($p < 0.001$).

Table 5b
% RI in HA plates[a] when compared with RI in control plates.

	Control	Ta37	TaΔTri5
Cellophane	+51.74 ± 3.16	0.00 ± 0.00	+52.15 ± 0.83
Dialysis	+59.61 ± 1.01	0.00 ± 0.00	+73.64 ± 0.89

Control = diameter of growth of the strain on plates without added HA.
Problem = diameter of growth of the strain on plates with added HA.
[a] % RI in HA plates = 100 × [(Control-Problem)/Control].

any of the collateral products detected in the *tri4*-disrupted mutant of *T. arundinaceum* (Malmierca et al., 2012). In addition, all the *tri* genes located in the "main cluster" of genes involved in HA biosynthesis, with the exception of the *tri4* gene, were expressed at a higher level than in the wild type strain. Even though *tri5* was disrupted, expression of the partial gene was increased in the mutant. This is an interesting difference in comparison to the *tri4*-disrupted transformant (Malmierca et al., 2012), which had reduced expression of the *tri4* and *tri5* genes, presumably as a response to the lack of an efficient processing of trichodiene, the product of the Tri5 protein, which must act as a negative feedback regulator of *tri5* expression. The absence of induction of the *tri4* gene in the TaΔTri5 strain can be explained by the lack of production of trichodiene, the substrate of Tri4. This suggests that in *Trichoderma*, only *tri4* is induced by its enzymatic substrate, and that the remaining *tri* genes are under separate controls, perhaps by Tri6 and/or Tri10, as seen in *Fusarium* (Tag et al., 2001; Peplow et al., 2003; Seong et al., 2009).

The expression of genes involved in other key steps of the terpene biosynthetic pathway was deeply altered in the *tri5*-disrupted mutant. Thus, it was observed that at 24 h of growth, the level of expression of *hmgR*, *dpp1*, and *erg9* genes was significantly increased in comparison with the wild type strain. In parallel, at this time of culture, HMGR activity was strongly increased in the TaΔTri5 mutant and the production of squalene and ergosterol were increased by 85% and 88% respectively. These results can be understood as a response of the cell to prevent the toxic accumulation of isoprenoid pyrophosphates, such as FPP, by redirecting it toward the production of farnesol and squalene/ergosterol (Fig. 8). The expression of *erg1* and *erg7* genes was not significantly affected in 24 h cultures, even when the level of ergosterol production was much higher than in the wild type strain, which might indicate that the level of expression of these two genes is not a bottleneck in ergosterol biosynthesis. However, as it has been previously described for *Saccharomyces cerevisiae*, the squalene epoxidase protein, encoded by the *ERG1* gene, would have a more important role as a flux controlling step in the ergosterol biosynthetic pathway (Veen et al., 2003), since this enzyme has a higher km value for its substrate and lower specific activity than the squalene synthase (encoded by the *ERG9* gene) and this could lead to an accumulation of squalene if the flux through the pathway exceeds a certain limit (Asadollahi et al., 2010).

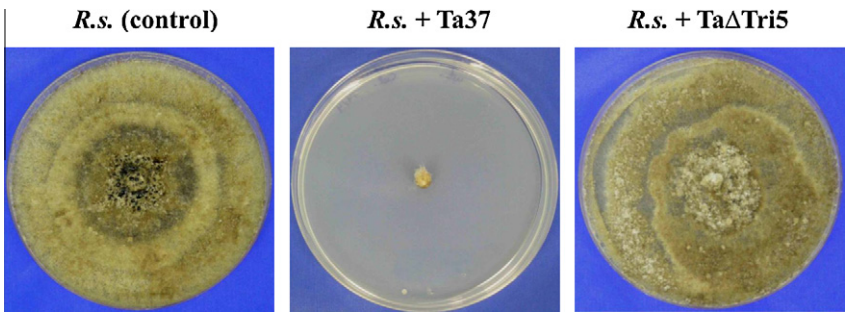


Fig. 6. Inhibition of *R. solani* (*R.s.*) growth by *T. arundinaceum* strains in a dialysis membrane assay. Photographs were taken after 6 days of incubation at 28 °C once the agar plugs inoculated with *R. solani* were placed in the middle of the plate, where previously a *T. arundinaceum* strain had been grown or not (control).

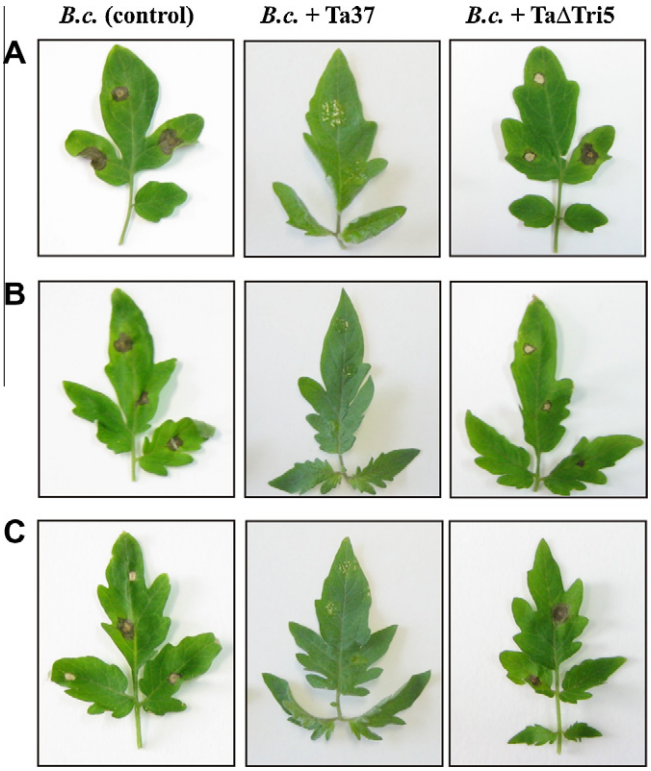


Fig. 7. Pathogenicity assays of *B. cinerea* on tomato leaves from three varieties (A) "Marmande" (B) "Tres Cantos" and (C) "Muchamiel". Leaves were inoculated with *B. cinerea* spores alone (*B.c.*) or in combination with broths from strains Ta37 or TaΔTri5. The experiment was performed on 12 leaves, incubated until 96 h, and repeated 3 times.

Table 6
Size of the *B. cinerea* lesions (in mm) on leaves from three tomato varieties measured at two different times after pathogen inoculation without (control) or with culture broth of the different *T. arundinaceum* strains used in this work. Data were analyzed by ANOVA (Analysis of variance) and the significant differences were determined by a Duncan Test with a $p < 0.05$ using the IBM SPSS Statistics 19 software.

	Control	Ta37	TaΔTri5
Tomato var. Marmande (n = 71):			
48 h	$5.13^{a} \pm 1.31$	$0.99^{c} \pm 1.38$	$1.46^{b} \pm 1.14$
72 h	$6.96^{a} \pm 2.02$	$1.16^{c} \pm 1.55$	$2.72^{b} \pm 1.86$
Tomato var. Tres cantos (n = 71):			
48 h	$4.58^{a} \pm 0.93$	$0.04^{c} \pm 0.20$	$1.38^{b} \pm 1.19$
72 h	$6.46^{a} \pm 1.73$	$0.29^{c} \pm 0.69$	$2.71^{b} \pm 1.71$
Tomato var. Muchamiel (n = 71):			
48 h	$4.76^{a} \pm 1.13$	$0.28^{c} \pm 0.56$	$1.49^{b} \pm 1.12$
72 h	$6.54^{a} \pm 2.15$	$0.58^{c} \pm 1.02$	$3.07^{b} \pm 2.02$

[a,b,c] For each tomato variety and each time point, values followed by different superscript letters are significantly different.

At 96 h, the level of squalene in cultures of the *tri5*-disrupted mutant was similar to that in the Ta37 strain, but the ergosterol level in the mutant was elevated, even though the difference with the wild type strain was less than that observed at 24 h. This would indicate that at this time point the accumulated squalene has been efficiently processed to ergosterol. The levels of *hmgR*, *dpp1*, and *erg9* gene expression and the level of HMGR activity were reduced in comparison with those detected at 24 h, probably as a result of the non-accumulation of squalene and/or FPP. The expression of *erg1* and *erg7* genes was also repressed in comparison with the wild type strain, even when the level of ergosterol was higher in the *tri5*-disrupted mutant, which led us to hypothesize that the expression of these two genes is not limiting for ergosterol biosynthesis at any of the assayed times. These results correlate well with a similar effect described in *S. cerevisiae*, where the attenuation in the expression of the squalene synthase encoding gene (*ERG9* gene) stimulated sesquiterpene production as a result of a redirection of the FPP pool (Paradise et al., 2008; Asadollahi et al., 2010).

The regulation of the mevalonate pathway in eukaryotes is complex, but HMGR and the FPP branch point represent two key regulation steps (Maury et al., 2005). The HMGR enzyme has been studied mainly in mammals, where it has been recently described that its endoplasmic reticulum associated degradation (ERAD) is regulated by a nonsterol signal, in the form of the geranylgeranyl moiety of a protein, and a sterologenic signal in the form of squalene (Leichner et al., 2011). In filamentous fungi, it is not known yet if HMGR enzyme degradation is regulated in a similar way. However, the increases in HMGR activity and in ergosterol level observed in the present work at 24 and 96 h of growth can be additionally explained as a way to avoid the accumulation of squalene, which might indicate the existence of a mechanism that regulates HMGR degradation by a similar way to that observed in mammals (Fig. 8). Nevertheless, additional studies would be needed to confirm this hypothesis.

In the present work we have found, as indicated above, that in 96 h old mycelia the HMGR activity was higher in the TaΔTri5 mutant (+23.9%) than in the Ta37 strain, even though the level of *hmgR* gene expression was lower (0.296, $p = 0.000$) in the *tri5*-mutated strain. This lack of correlation between the level of gene expression and the activity of its corresponding enzyme activity might be explained not only by the regulation of HMGR stability discussed above, but by other phenomena as well, such as stability of the mRNAs, ribosome occupancy, and protein abundance and stability (Maier et al., 2009).

The differences in the chitinase activity observed in the TaΔTri5 strain, which showed similar or slightly lower activity level as in Ta37, in comparison with the TaΔTri4 mutant which had increased activity, can perhaps be explained by the fact that even though these mutants are affected in the same biosynthetic pathway, they show important differences. The *tri4* disrupted mutant produced, as collateral products, 12,13-epoxytrichoene-2-ol derivatives of trichodiene that may have some regulatory function on the induction of chitinase gene expression. These compounds, or other trichothecene intermediates, are not produced by the TaΔTri5 strain, and therefore would not show any stimulatory effect on chitinase activity.

The antifungal activity of Ta37 and TaΔTri5 against the two phytopathogenic fungi used in this study was notably different between the two strains, with TaΔTri5, which lacks HA, causing less inhibition of *B. cinerea* growth than Ta37. If HA was entirely responsible for the observed growth inhibition by Ta37, no inhibition would be expected with the TaΔTri5/cellophane conditions. Instead, a 73.5% inhibition was seen. This suggests that HA inhibits the growth of *B. cinerea* by 26.5% and other metabolites and/or enzymes are responsible for 73.5% of the observed inhibition, of which 11.2% [(100–88.8%) Table 5a] might be due to a higher MW metabolite or enzyme. These calculations suggest that TaΔTri5 on dialysis membrane should exhibit an inhibition of 62.3% (88.8–26.5%). Instead, we have seen a lower inhibition of 46.4%. Perhaps the higher inhibition on dialysis membranes with the wild type strain is due to a synergistic interaction of HA with other metabolites.

Results on *R. solani* showed that HA contributes only about a 5% inhibition [(100–95.2%) see Table 5a] when using the cellophane assay. On dialysis membranes, the inhibition was 67.7% when TaΔTri5 strain was used, again suggesting that HA may interact synergistically with other metabolites to inhibit *R. solani* growth.

It is also possible that in this assay the difference between these two strains might also be due to the lack of production of some kind of compound other than HA in the TaΔTri5 mutant, although this compound needs to be discovered. The reduction in the bio-

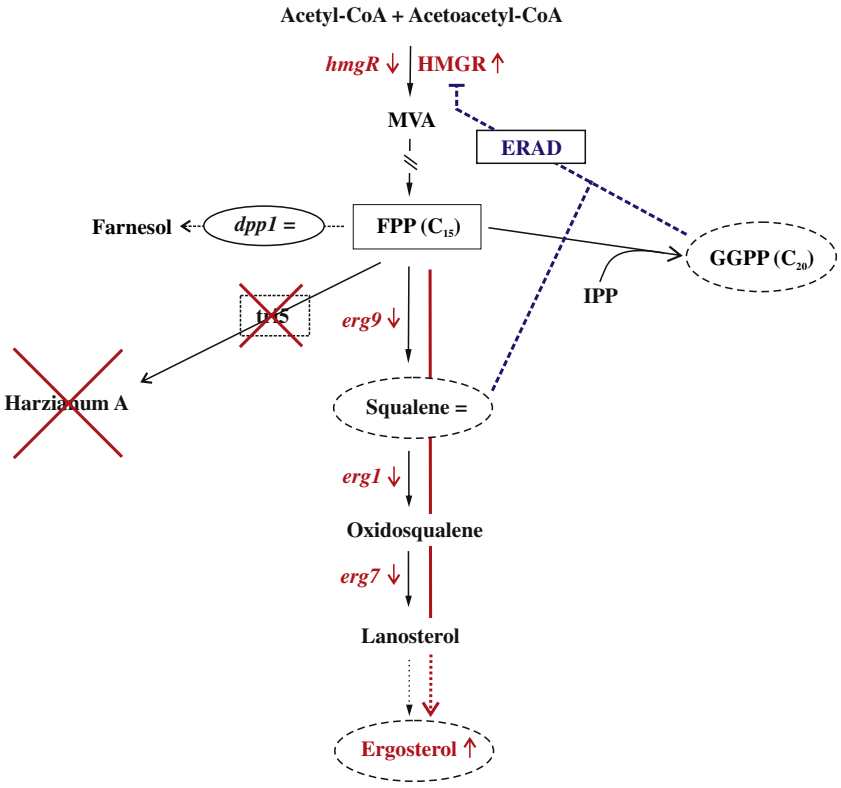


Fig. 8. Schematic representation of the terpene biosynthetic pathway, indicating in red those changes observed in the TaΔTri5 mutant in comparison with the Ta37 strain in mycelia grown for 96 h. Blue dotted lines indicate the signals described in mammals that are needed for ERAD (endoplasmic reticulum associated degradation) of HMGR enzyme and that are overcome by the TaΔTri5 mutant (Leichner et al., 2011). IPP: isopentenyl diphosphate, GGPP: geranylgeranyl diphosphate.

control activity in TaΔTri5 *vs.* Ta37 was also confirmed by the reduction in the ability to inhibit the germination of *B. cinerea* conidia, with broths of Ta37 having greater inhibition than those of the *tri5*-disrupted mutant.

The observed reduction in the biocontrol activity on membrane assays by the *tri5* gene disrupted mutant was greater than that detected with a *tri4*-disrupted mutant (Malmierca et al., 2012). This may be due to the total loss of HA in the TaΔTri5 mutant, as well as the production of several intermediates in the biosynthesis of trichothecenes and collateral compounds in the *tri4*-disrupted transformant. These compounds, together with the higher level of chitinase production, could contribute to the greater biocontrol activity shown by the *tri4*-disrupted mutant in comparison with the *tri5*-disrupted transformant analyzed in the present work.

The lesions observed in tomato leaves infected with *B. cinerea* conidia and separately treated with culture broths from the two *Trichoderma* strains used in this work indicate that although HA is an important factor that limits the development of *B. cinerea* disease, other compound/s produced by *Trichoderma* must be involved as well. This is supported by the results using the broth of TaΔTri5 whereby there was a reduction in the size of *B. cinerea* lesions on the tomato leaves. If HA was acting alone, the lesions should have been the same size in both the TaΔTri5 and control treatments. This is also supported by biocontrol assays on membranes which showed that in addition to HA, *T. arundinaceum* produced other compound/s, not yet identified, that may synergistically contribute to the antifungal activity of this metabolite.

5. Conclusions

Trichothecene biosynthesis plays an important role in fungal physiology. In general, these compounds show toxicity to plants as well as to animals. This study has shown that Harzianum A, produced by *T. arundinaceum*, is toxic to some phytopathogenic fungi and plays a role in antifungal activity. The presence of HA also contributes to the regulation of intracellular levels of FPP, as well as the levels of intermediates involved in terpene biosynthesis and end products such as ergosterol. The production of trichothecenes by selected fungi is obviously important to their success in their external environment as well as providing for a delicate balance in their internal environment.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fgb.2013.02.001.

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